0163-4984/02/8603-0237 $12.75

Renal Copper as an Index of Copper Status in Marginal Deficiency

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Received October 29, 2001; Accepted November 21, 2001

ABSTRACT

Marginal copper (Cu) deficiency is difficult to study, in part because its effects may be small, but also because feeding of a deficient diet may not cause a discernable change in Cu status. The key to resolution of effects may be in the choice of Cu status index. In this study, liver Cu concentration, a commonly used index of Cu status, was compared with activity of ceruloplasmin (CP), a circulating Cu-dependent enzyme, and kidney Cu concentration for their utility in resolving effects of marginal Cu deficiency. Seventy male, weanling rats were fed diets containing, nominally, 0, 1.5, 3, 4.5, or 6 mg Cu/kg diet for 5 wk. All three indices showed strong depression with severe deficiency (dietary Cu=0), but were relatively weak in their ability to distinguish between animals fed marginally deficient diets when compared by group statistics (ANOVA). Further, group statistics revealed no effect of marginal deficiency on six other variables known to change with severe Cu deficiency: heart weight/body weight, hematocrit, red cell distribution width, neutrophil count, glycated hemoglobin, and platelet count. To take into account interanimal variation, the three putative indices were plotted against these six variables and linear regression was performed on points representing marginally deficient rats. None of the variables showed significant regression with liver Cu or serum ceruloplasmin, but three showed significant regression with kidney Cu. These findings indicate that kidney Cu is preferable to liver Cu or ceruloplasmin as an index of Cu status in marginal deficiency and that linear regression is a possible way of testing for effects of marginal Cu deficiency, especially when effects are subtle.

Index Entries: Copper deficiency; kidney; liver; ceruloplasmin; heart; erythrocyte; neutrophil; glycation; platelet.

INTRODUCTION

One of the ultimate objectives of nutritional experiments in animals is to apply the knowledge learned to humans. To do so, the conditions used for animals must parallel those expected to be found in humans. Many, if not most, studies that examine the essentiality of trace elements (e.g., copper) in animals use diets that are severely deficient. Although such diets are useful for studying the absolute need for an element, they may not compare to the reality of diets in humans, which may be only marginally low in the trace element of interest.

An important aspect of studying any type of deficiency is to find an appropriate index of nutritional status. A commonly used index of copper status in animals subjected to severe copper deficiency is liver copper. In a preliminary study of marginal copper deficiency, liver copper did not change sufficiently at two marginally deficient dietary copper levels to be useful as a copper status index. However, kidney copper concentration appeared to be more promising as an index in that it showed a more uniform gradation with marginal dietary copper and better correlation with effects already known to change with severe deficiency.

The present study examined the influence of a relatively tightly graded range of dietary copper levels on three indices of copper status. [liver copper, kidney copper, and serum ceruloplasmin-[a Cu-dependent enzyme *(1)*]] and, secondly, to determine whether any of these indices could be used to discern effects of marginal copper deficiency on variables already known to be affected by severe deficiency.

MATERIALS AND METHODS

Animals and Diets

Seventy male weanling Sprague-Dawley rats were divided into five equal, weight-matched groups. Each group was fed *ad libitum* one of five purified diets that contained, nominally, 0, 1.5, 3, 4.5, or 6 mg Cu/kg diet. Copper-adequate and copper-deficient diets were composed of 940.0 g of copper-free, iron-free basal diet (catalog #TD 84469, Teklad Test Diets, Madison, WI, USA), 50.0 g of safflower oil (Hollywood Foods, Los Angeles, CA, USA), and 10.0 g of a copper–iron mineral mix per kilogram of diet. The basal diet was a casein (200 g/kg), sucrose (386 g/kg), cornstarch (295 g/kg)-based diet containing all known essential vitamins and minerals except for copper and iron *(2)*. The mineral mix contained cornstarch (Argo, CPC Food Service, Englewood Cliffs, NJ, USA) and iron with or without copper, and it provided 0.22 g of ferric citrate (16% Fe) (J.T. Baker Chemical Co., Phillipsburg, NJ, USA) and either 0, 6, 12, 18, or 24 mg of added $CuSO_4 \cdot 5H_2O$ (J.T. Baker Chemical Co.) per kilogram of diet. These formulations were intended to provide a severely copper-deficient diet

(CuD) containing only copper present in the basal diet, copper-deficient diets with 1.5, 3, or 4.5 mg Cu/kg diet, and a copper-adequate diet (CuA) containing 6 mg Cu/kg diet. Dietary analyses of three samples from each diet indicated that the copper concentrations of the diets were 0.27 ± 0.07 (SD), 1.43 ± 0.03, 2.92 ± 0.05, 4.27 ± 0.17, and 6.15 ± 0.23 mg Cu/kg diet.

Analysis of dietary copper was initiated by dry-ashing of the tissue sample *(3),* followed by dissolution in aqua regia and measurement by atomic absorption spectroscopy (model 503, Perkin Elmer, Norwalk, CT, USA). Validation of the assay method was provided by simultaneous assays of a corn kernel reference standard (#8413, National Institute of Standards and Technology, Gaithersburg, MD, USA), and a dietary reference standard (HNRC-2) that was developed by the Grand Forks Human Nutrition Research Center.

Blood and Tissue Analysis

After consumption of their respective diets for 5 wk, rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (65 mg/kg body weight, Sigma, St. Louis, MO USA). Blood was withdrawn from the inferior vena cava and divided into aliquots for blood cell counting (EDTA treated) and serum assays (samples allowed to clot at room temperature). Hearts and kidneys were collected for copper assays.

Hematocrits, red blood cell distribution widths, platelet counts, and neutrophil counts were determined on EDTA-treated blood by use of a cell counter (Cell-Dyn model 3500CS, Abbott Diagnostics, Santa Clara, CA, USA).

Glycated hemoglobin was measured on EDTA-treated blood by using the Glycated Hemoglobin (Hb A_1) kit (Procedure No. 441) from Sigma Diagnostics (St. Louis, MO USA), which utilizes a cation-exchange resin to separate Hb A from Hb A_1 and spectrophotometric measurement (415 nm) of each fraction. Hb A_1 is expressed as a percentage of total Hb A.

Ceruloplasmin was measured in serum by its *p*-phenylenediamine oxidase activity *(4).*

Livers and kidneys were lyophilized and digested with nitric acid and hydrogen peroxide *(5).* Organ copper concentration was determined by inductively coupled plasma–emission spectroscopy (Jarrell Ash, model 1140, Waltham, MA, USA). Simultaneous assay of a reference sample (#1577a, bovine liver, National Institute of Standards and Technology, Gaithersburg, MD, USA) fell within the certified range for copper, thus validating our assay method.

Statistical Analysis

Variation of proposed copper status indices (liver Cu, kidney Cu, serum ceruloplasmin) and of test variables (heart weight, hematocrit, red cell distribution width, platelet count, neutrophil count, glycated hemoglobin) with diet were analyzed by analysis of variance (ANOVA) on

ranks and subsequent pairwise comparison by Dunn's method (SigmaStat Statistical Software, Jandel Scientific, San Rafael, CA). Sample numbers (listed in Tables 1 and 2), which originated at 14 per group, were reduced by 1 rat death in the group fed 1.5 mg Cu/kg diet, by the odd statistical outlier in some groups and, in the case of ceruloplasmin measurements, by insufficient serum for the measurement in 20 of the 70 rats.

To determine whether a statistically significant relationship existed between a copper status index and a test variable for marginal deficiencies, first-order regression analysis was done on composite plots of data for rats that were fed nominal diets of 1.5, 3, 4.5, and 6 mg Cu/kg diet (SigmaPlot Scientific Graphing Software, Jandel Scientific, San Rafael, CA). The resulting correlation coefficients were compared to tabulated critical values for statistical significance *(6).*

RESULTS

The variation of the putative indices of copper status (liver Cu, kidney Cu, and ceruloplasmin) with dietary copper is shown in Table 1. Statistical analysis indicates that these indices respond similarly to alteration of dietary copper, showing little change with dietary copper at high concentrations and more change at lower concentrations. Visual examination of the figures suggests that kidney copper provides the most evenly graded response and, therefore, offers the best potential for differentiating effects of copper deficiency when the deficiency is not severe.

Variation of six test variables with dietary copper is given in Table 2. Statistical analysis (ANOVA) indicates that, although all variables show significant differences between rats fed severely copper-deficient diets and those fed all other diets, there were no statistical differences among these variables when dietary copper was 1.5 mg/kg or greater.

Test variables are plotted as a function of the three copper status indices in Figs. 1–6. Each point represents data from a single rat. In order to test for the effects of marginal copper deficiency, first-order regression was performed on all data except for that from rats fed the most severely deficient diet. When liver copper or ceruloplasmin was used as the copper status index, none of the test variables showed a statistically significant regression coefficient. Using kidney copper as the copper status index, three of the test variables (heart weight, red cell distribution width, and neutrophil count) showed statistically significant regression coefficients.

DISCUSSION

The objective of this study was to determine whether variables known to be affected by severe dietary copper deficiency could be shown to be affected by marginal copper deficiency. This was done by feeding rats rel-

Table 1
Effect of Graded Dietary Copper on Putative Copper Status Indices

Dietary copper mg kg^{-1}	Copper index		
	Liver Cu μg g^{-1}	Kidney Cu μg g^{-1}	Ceruloplasmin mg dL^{-1}
0	1.4 ± 0.3 (13) [a]	10.0 ± 0.2 (14) [a]	4.9 ± 0.1 (12) [a]
1.5	12.1 ± 0.7 (13) [ab]	15.6 ± 0.5 (13) [ab]	23.3 ± 4.6 (9) [ab]
3	14.4 ± 0.3 (13) [bc]	19.3 ± 0.7 (14) [bc]	56.8 ± 7.4 (10) [b]
4.5	14.4 ± 0.3 (14) [bc]	21.6 ± 0.6 (13) [c]	59.6 ± 7.9 (13) [b]
6	15.8 ± 0.6 (13) [c]	22.9 ± 1.1 (14) [c]	40.8 ± 4.6 (5) [b]

Note: Values are means ± SEM *(n)*.
[a,b,c] Values in a column with no common superscript are different ($p<0.05$).

atively small graded increments of copper and then assessing their copper status and changes in variables known to be affected by severe copper deficiency. It is apparent that, although use of group statistics (specifically, ANOVA) showed some effects of marginal deficiency on variables most directly related to copper status (i.e., liver and kidney Cu, Table 1), ANOVA revealed no effects of marginal copper deficiency on variables that are less directly related to copper (Table 2). This may be because the variables are not affected by marginal copper deficiency or because the analytical or statistical methods cannot resolve the differences.

Considering the latter possibility, we reasoned that characterizing an animal's copper status by the diet they eat may not provide sufficient resolution. Despite the fact that two animals eat the same diet, copper status may differ between them, depending on a variety of aspects related to biological variation. This is illustrated in Figs. 1–6, where copper status indicators of rats eating the same diet vary and, in fact, overlap with those of rats eating another diet. Because of this, mathematical regression against copper status indices was perceived as offering better potential (than comparing a group of animals fed one diet against one fed another diet) for discerning the effects of marginal copper deficiency.

Six representative variables known to be affected by severe copper deficiency—heart weight *(7)*, hematocrit *(8)*, red cell distribution width *(8)*, neutrophil count *(9)*, platelet count *(10)*, and glycated hemoglobin *(11)*—were plotted against three variables that may be regarded as copper status indices—liver and kidney copper concentrations and serum ceruloplasmin. Regression analysis performed on all points except for those from rats fed severely deficient diets showed no significant regression for any of the variables against liver copper concentration or ceruloplasmin, but did show significant regression coefficients for three of the variables plotted against kidney copper concentration. Thus, of the three indices, only kidney copper directed us to three potentially useful directions of study of

Table 2
Effect of Graded Dietary Copper on Variables Known to be Affected by Severe Dietary Copper Deficiency

Dietary copper	Variable					
mg kg^{-1}	Heart wt	Hematocrit	RDW *	Neutrophil count	Platelet count	Hb_{A1c}
	mg g^{-1}	%	%	μL^{-1} ($x10^3$)	μL^{-1} ($x10^3$)	%
0	5.24 ± 0.22 (14) [a]	25.2 ± 1.3 (13) [a]	27.0 ± 1.7 (13) [a]	0.294 ± 0.038 (12) [a]	1026 ± 49 (13) [a]	2.62 ± 0.06 (14) [a]
1.5	3.74 ± 0.07 (13) [b]	39.7 ± 0.8 (13) [b]	15.9 ± 0.3 (13) [ab]	0.599 ± 0.068 (13) [b]	834 ± 27 (13) [ab]	2.07 ± 0.02 (13) [b]
3	3.59 ± 0.05 (13) [b]	41.0 ± 0.8 (14) [b]	14.8 ± 0.4 (14) [b]	0.604 ± 0.058 (14) [b]	801 ± 43 (14) [b]	2.08 ± 0.02 (14) [b]
4.5	3.60 ± 0.07 (14) [b]	40.0 ± 1.1 (13) [b]	14.8 ± 0.3 (13) [b]	0.653 ± 0.057 (13) [b]	844 ± 18 (12) [ab]	2.06 ± 0.01 (14) [b]
6	3.55 ± 0.07 (14) [b]	41.2 ± 0.8 (14) [b]	14.6 ± 0.3 (13) [b]	0.701 ± 0.075 (13) [b]	809 ± 16 (13) [b]	2.07 ± 0.02 (14) [b]

Note: Values are means ± SEM *(n)*.
* Red blood cell distribution width [100 × (SD of red blood cell volume)/(mean red blood cell volume)].
[a,b] Values in a column with no common superscript are different ($p<0.05$).

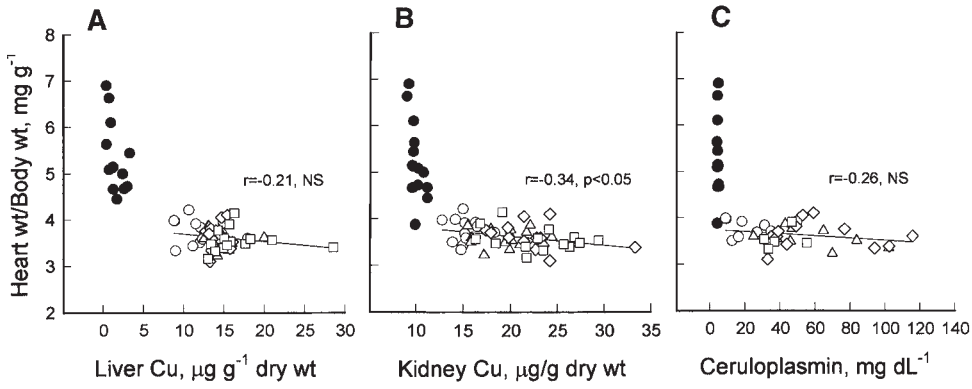

Fig. 1. Heart weight/body weight ratio as a function of liver Cu concentration **(A),** kidney Cu concentration **(B),** and ceruloplasmin concentration **(C).** Symbols represent values from rats fed diets with nominal copper concentrations of 0 (●), 1.5 (○), 3 (△), 4.5 (◇), and 6 mg/kg (□). The correlation coefficient *(r)* and *p*-value are calculated from the linear regression on all points except for those from the most severely deficient diet.

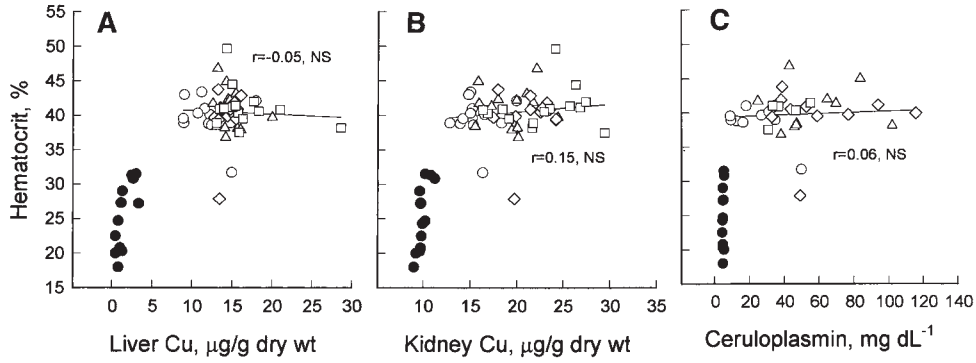

Fig. 2. Hematocrit as a function of liver Cu concentration **(A),** kidney Cu concentration **(B),** and ceruloplasmin concentration **(C).** Symbols represent values from rats fed diets with nominal copper concentrations of 0 (●), 1.5 (○), 3 (△), 4.5 (◇), and 6 mg/kg (□). The correlation coefficient *(r)* and *p*-value are calculated from the linear regression on all points except for those from the most severely deficient diet.

marginal copper deficiency. The first indicates that a change in heart weight, and thus molecular changes that contribute to it, is detectable in marginally copper-deficient rats. This is supported by the findings of Wildman et al. *(12),* which indicated that, despite the absence of changes in copper status indices, the feeding of a marginally deficient diet leads to ultrastructural changes in the heart, some of which could lead to hypertrophy. The present study suggests that if a more sensitive copper status index were used, the changes could have been more persuasively linked to

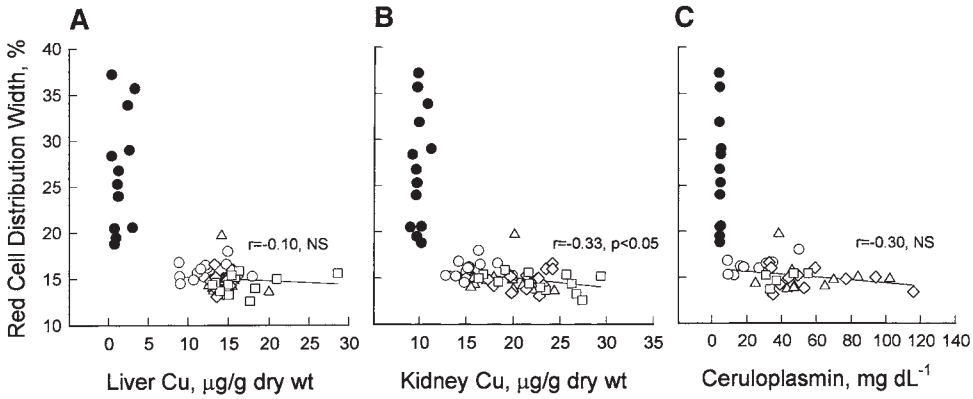

Fig. 3. Red blood cell distribution width [100 × (SD of red blood cell volume)/(mean red blood cell volume)] as a function of liver Cu concentration **(A),** kidney Cu concentration **(B),** and ceruloplasmin concentration **(C).** Symbols represent values from rats fed diets with nominal copper concentrations of 0 (●), 1.5 (○), 3 (△), 4.5 (◇), and 6 mg/kg (□). The correlation coefficient *(r)* and *p*-value are calculated from the linear regression on all points except for those from the most severely deficient diet.

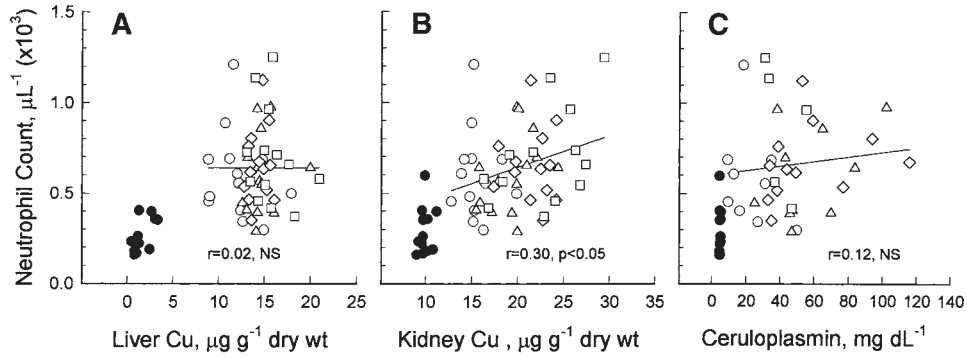

Fig. 4. Neutrophil count as a function of liver Cu concentration **(A),** kidney Cu concentration **(B),** and ceruloplasmin concentration **(C).** Symbols represent values from rats fed diets with nominal copper concentrations of 0 (●), 1.5 (○), 3 (△), 4.5 (◇), and 6 mg/kg (□). The correlation coefficient *(r)* and *p*-value are calculated from the linear regression on all points except for those from the most severely deficient diet.

a change in copper status. Similarly, the changes in red cell distribution width and neutrophil count direct us to the study of, respectively, red cell destruction/erythropoesis and immune function in marginal copper deficiency. Regarding the latter, Hopkins and Failla *(13)* observed changes in leukocyte function in rats fed a marginally deficient diet without changes in traditional copper status indices. However, they did observe a depres-

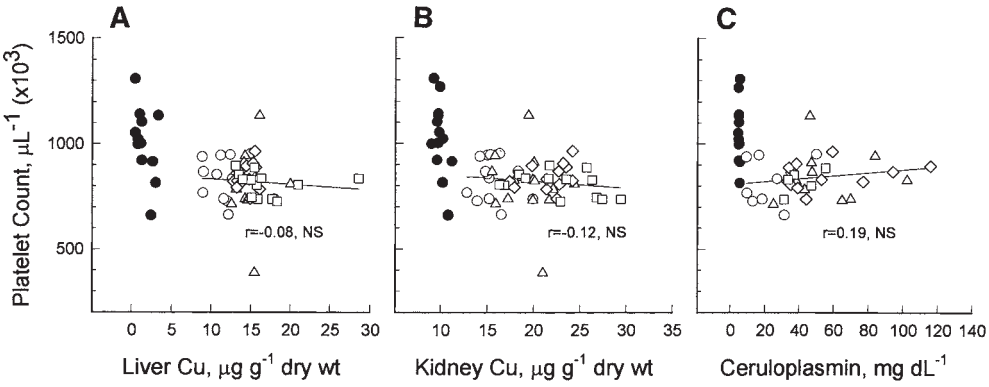

Fig. 5. Platelet count as a function of liver Cu concentration **(A),** kidney Cu concentration **(B),** and ceruloplasmin concentration **(C).** Symbols represent values from rats fed diets with nominal copper concentrations of 0 (●), 1.5 (○), 3 (△), 4.5 (◇), and 6 mg/kg (□). The correlation coefficient *(r)* and *p*-value are calculated from the linear regression on all points except for those from the most severely deficient diet.

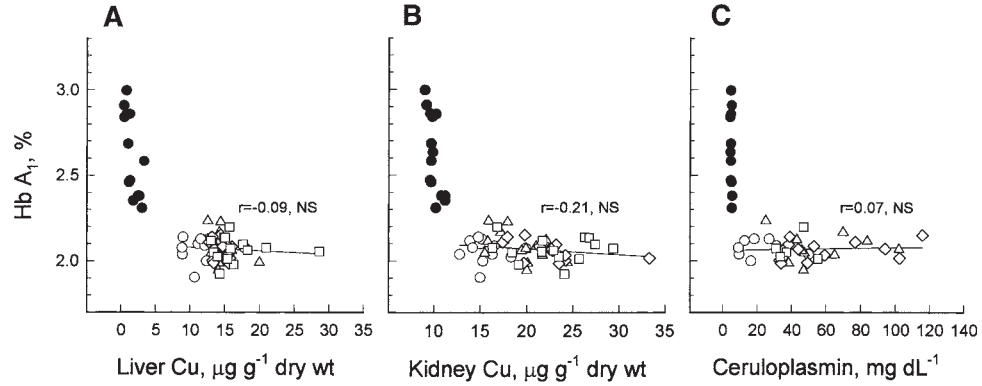

Fig. 6. Glycated hemoglobin (Hb A_1) as a function of liver Cu concentration **(A),** kidney Cu concentration **(B),** and ceruloplasmin concentration **(C).** Symbols represent values from rats fed diets with nominal copper concentrations of 0 (●), 1.5 (○), 3 (△), 4.5 (◇), and 6 mg/kg (□). The correlation coefficient *(r)* and *p*-value are calculated from the linear regression on all points except for those from the most severely deficient diet.

sion of brain copper concentration. Perhaps brain copper is akin to kidney copper in the present study in that it provides improved resolution of copper status.

From the above observations, several general conclusions are possible:

1. Kidney copper concentration provides a good index for resolving potential effects of marginal copper deficiency in laboratory animals. Standard indices of copper status, such as liver copper

concentration and serum ceruloplasmin, which, although suitable for establishing the presence of severe Cu deficiency, are of less use in discerning effects of marginal deficiency.
2. Analysis of variance among groups subjected to different dietary copper concentrations is ineffective for resolving effects of marginal copper deficiency. Mathematical regression of a suspected copper-dependent variable against a reliable copper status index provides a good potential method for discerning effects of marginal copper deficiency. Some useful corollaries also follow from these findings.
3. Although kidney copper concentration *per se* will not be a useful copper status index for human studies, because its measurement is invasive, its use here establishes the paradigm of using mathematical regression that may also be applied to a suitable human index of copper deficiency, when it is found.
4. Because of the subtlety of the effects of marginal copper deficiency, large numbers of subjects will be required to discern additional effects of marginal copper deficiency in animals or humans.

ACKNOWLEDGMENTS

The author acknowledges the technical assistance of Gwen Dahlen and Pete Leary, animal care by Debbie Merrill, and diet mixing by Karin Tweton.

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